Single High-Energy Impact Load Causes Posttraumatic OA in Young Rabbits via a Decrease in Cellular Metabolism

Joseph Borrelli Jr.,1,2 Matthew J. Silva,1 Melissa A. Zaegel,1 Carl Franz,1 Linda J. Sandell1

1Department of Orthopaedic Surgery, Washington University School of Medicine, Barnes–Jewish Hospital, St. Louis, Missouri, 2Department of Orthopaedic Surgery, University of Texas–Southwestern Medical Center, 1801 Inwood Road, W4A 312, Dallas, Texas 75390-8883

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ABSTRACT: Articular cartilage deterioration commonly occurs following traumatic joint injury. Patients with posttraumatic osteoarthritis (PTA) experience pain and stiffness in the involved joint causing limited mobility and function. The mechanism by which PTA occurs has not been fully delineated. The goal of this study was to determine if a single high-energy impact load could cause the development of PTA in 3-month-old NZ White rabbits. Each rabbit underwent the application of a single, rapid, high-energy impact load to the posterior aspect of their right medial femoral condyle using a previously validated mechanism. At regular intervals (0, 1, 6 months) the injured cartilage was harvested and analyzed for the presence of PTA. Each specimen was assessed histologically for cell and tissue morphology and chondrocyte metabolism, including BMP-2 production and synthesis of extracellular matrix (type II procollagen mRNA). Cartilage from the contralateral sham limb, as well as uninjured cartilage from the experimental limb served as internal controls for each animal. Significant changes were found in the morphology of the cartilage including proteoglycan loss along with decreased BMP-2 and type II procollagen mRNA staining. These findings confirm that a single high-energy impact load can cause the development of PTA by disrupting the extracellular matrix and by causing a decrease in chondrocyte metabolism. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:347–352, 2009

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Posttraumatic osteoarthritis (PTA) occurs when articular cartilage deteriorates in response to injury. Twelve percent of patients with symptomatic OA of the lower extremity joints are felt to be suffering from PTA.1 As a result, PTA has been estimated to have an aggregate US financial burden of $3 billion annually or 0.15% of the total U.S. health care direct cost outlay. In addition to these financial costs, PTA is associated with disability, pain and joint stiffness, and an overall decrease in total U.S. health care direct cost outlay. In addition to these financial costs, PTA is associated with disability, pain and joint stiffness, and an overall decrease in function.2–5 The development of PTA has been associated with ligamentous injury and intraarticular fracture. Although surgical treatment schemes have been developed to repair these injuries, these treatment schemes have not been completely successful in preventing the development of PTA.

Investigations into the effects of mechanical injury on articular cartilage have been ongoing for some time. These studies have shown the development of degenerative changes can occur in articular cartilage following mechanical injury. As a result, most investigators now believe that primary cartilage damage plays an important role in the development of PTA following joint injury.6–9 However, the exact mechanism by which a mechanical injury stimulates the deterioration of articular cartilage has not been fully delineated.

It is unclear whether these changes are the direct result of the mechanical trauma or the result of the injury stimulating an active process within the cartilage that causes cartilage degeneration. Finding the answer to this question will lead to the development of methods to mitigate the detrimental effects of mechanical injury on articular cartilage.

Correspondence to: Joseph Borrelli, Jr. (T: 214-645-3336; F: 214-645-3350; E-mail: joseph.borrelli@utsouthwestern.edu)
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METHODS
Using a previously validated in vivo open joint injury model, the effects of a single impact load on the development of PTA were investigated. Twenty-two New Zealand White (NZW) rabbits (3 kg; average age 3 months), underwent impact loading of the right medial femoral condyle using a pendulum type device, as previously described.6,10 The surgical procedure was slightly altered to provide increased stabilization of the hind limb during impact. One 1.6-mm Kirschner wire was passed medial to lateral through the femoral condyles and was fixed to the underlying polyethylene block to prevent movement of the limb at the moment of impact. A second Kirschner wire was passed posterior to anterior through the proximal tibia and into the polyethylene to provide stabilization of the tibia. To avoid fracturing the distal femur at the time of impact, the previously utilized Kirschner wire, placed posterior to anterior through the distal femur, was eliminated.10 To investigate the potential for a dose-dependent response, the right femoral condyle was struck with either a Low impact load or a High impact load. The group of animals that received the Low impact load had a mass of 4,350 gr attached to the midpoint of the pendulum arm, while the High impact load had a mass of 4,900 gr attached. These two different masses were used to create impact loads of approximately 70% (Low impact) and 90% (High impact) of the fracture threshold for the medial femoral condyles. The fracture threshold of the medial femoral condyle was determined prior to the start of this investigation using a biconcave impactor, a materials testing machine (Instron, Norwood, MA), and cadaveric rabbit limbs from similar age and size animals (unpublished data). Intraarticular fractures were avoided in order to minimize the addition of confounding factors associated with fractures. Once the condyle was struck,
the end of the impacter area was colored with a surgical marker and touched to the impacted area. The position of the impacted area was then localized relative to the medial, lateral, proximal, and distal aspects of the condyle, and these measurements were recorded. At the time of specimen harvest, these measurements were used to accurately identify the position of the impacted cartilage. For each rabbit, the left leg underwent a sham operation complete with an arthrotomy and touching of the impacter to the articular surface without application of load, and therefore served as an internal control for each of the investigations. Post-impact, the animals were allowed free cage activities, food ad libitum, and were euthanized at 0, 1, and 6 months. All procedures were approved by our Animal Studies Committee. The distribution of animals by experimental outcome is shown in Table 1.

**Histological Assessment**

To assess the initial changes within the injured cartilage as a result of the single impact load, two bone and cartilage specimens from each of the impact load groups at time zero were assessed (n = 4) histologically. Cartilage specimens from three rabbits in each of the two impact load groups and from each of the three time points were evaluated (n = 18). These evaluations included routine histology to assess cartilage morphology using hematoxylin and eosin (H&E). Safranin-O staining was used to assess the proteoglycan content of the extracellular matrix. Immunohistochemistry techniques as described by Fukui et al. were used to assess changes in BMP-2 production. In situ hybridization was used to assess procollagen type II mRNA synthesis and 18S ribosomal RNA content, and both were used as an indication of chondrocyte metabolism.

Using the damage scoring scale of Lewis, time zero cartilage and bone specimens were used to grade the severity of the injury. Grade 0 indicates that there was no disruption of the cartilage surface. Grade 1 indicates that minimal surface damage, such as a single fissure, was present; Grade 2 indicates that additional fissuring with interspersed regions of intact matrix was present; and Grade 3 indicates a complete disruption of the cartilage surface. At the time of euthanasia, the femoral condyle of each of the hind limbs was harvested. Using a bone cutting microtome (Isomet Low Speed Saw, Buehler, Lake Bluff, IL), the medial femoral condyles were fixed in 4% paraformaldehyde for 4 h at room temperature or overnight at 4 °C. Following fixation, the specimens were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Hematoxylin and Safranin-O staining were performed on consecutive slices of each specimen in the standard fashion. Subsequently, specimens from 1-month-old and 6-month-old rabbits were used to assess the progression of these initial changes, including changes in the appearance of the cartilage matrix and changes in the appearance, number, and position of the chondrocytes according to the work of Mankin et al.

**Immunohistochemistry**

For BMP-2 assessment, the paraffin-embedded cartilage specimens were cut into 6-μm sections. Anti-human BMP-2 goat polyclonal antisera (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect BMP-2, which was visualized with the streptavidin (Sigma, St. Louis, MO) and DAB from BrDu staining kit (Zymed Laboratories, Inc., South San Francisco, CA). Donkey anti-goat (Santa Cruz Biotechnology) secondary antibody was used to localize BMP-2. For control staining, nonimmune serum was used in place of the primary antibody.

### In Situ Hybridization to mRNA

Synthesis of type II procollagen was measured directly by in situ hybridization to mRNA within the chondrocytes. Briefly, cRNA (riboprobes) were generated from cDNA plasmids encoding for a fragment of type II procollagen (bovine IIB N-propeptide). This riboprobe has previously been shown to be specific for rabbit mRNA and does not cross react with other similar collagens. Radiolabeled riboprobe was added to hybridization buffer and hybridized at 53–58 °C. Unbound probe was hydrolyzed with RNase A, and final washes were carried out at high stringency (53–58 °C, 2 × SSC/50% formamide). Slides were then exposed to IsoMax Autoradiography/Xray film (SciMart, St. Louis, MO) for 3 days to approximate signal strength and prepared for autoradiography by dipping in photographic emulsion. The slides were dried, sealed, and kept in a dark box for 72 h at 4 °C.

**Table 1.** Demonstrates How Specimens from Each of the Rabbits were Utilized in the Study Relevant to Impact Load and Time from Injury

<table>
<thead>
<tr>
<th>Impact load</th>
<th>0 Months</th>
<th>1 Month</th>
<th>6 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Histology; cellular viability; immunohistochemistry; in situ hybridization</td>
<td>Acute injury assessment according to Lewis et al.</td>
<td>Histology to include degenerative changes (Mankin et al.); cellular viability; immunohistochemistry; in situ hybridization</td>
</tr>
<tr>
<td>4,350 gr (Low)</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>4,900 gr (High)</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Totals</td>
<td>6</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>
Sections were then counterstained by routine methods with Mayer’s Hematoxylin. Bright field and dark field microscopy was performed using a Nikon E800 microscope.

RESULTS
The addition of different masses to the pendulum arm resulted in the creation of two different impact groups (Low impact group and High impact group). The Low impact group received an average impact force of $744 \pm 90$ N, while the High impact group received a significantly greater impact force of $815 \pm 93$ N ($p = 0.008$). For each of these groups, the average time to peak impact was $18 \pm 2$ ms. The area of impact was $7.29 \pm 0.91$ mm$^2$.

Injury
Little gross evidence of cartilage injury was evident at the time of impact. Histologically, according to the damage scoring scale of Lewis et al., the Low impact group typically had grades of injury of 0 or 1; the High impact group typically had grades of injury of 2 and 3 (Fig. 1). Both of the impact groups showed further matrix deterioration over time. In addition to the worsening overall appearance of the cartilage, extensive chondrocyte clusters were found in the deep zone of the impacted cartilage, particularly at the 6-month time point. Many empty lacunae remained within the superficial and middle zones of the impacted cartilage at 6 months. Using the evaluation scheme of Mankin for evaluating osteoarthritis, each of the impact specimens had some degree of alteration in articular structure that was evident grossly (Fig. 2) and histologically. Obvious loss of Safranin-O staining and chondrocyte cloning was observed. These changes were most obvious in the High impact load, 6-month specimens as compared to the time 0- and 1-month specimens (Figs. 3A, 4A, 5A).

Molecular Analysis
At 0 time, both impacted and nonimpacted specimens showed the same result. There was strong Safranin-O staining and staining for BMP-2 within the cells, but not in the surrounding extracellular matrix (ECM) (Fig. 3A,B). High levels of type II procollagen mRNA were also observed (Fig. 3C).

At 1 month, significant differences were observed between the impacted areas and the surrounding cartilage. In impacted areas, Safranin-O staining was reduced and persisted primarily only in the immediate vicinity of the chondrocytes. Areas outside of the impact zone appeared to have somewhat less Safranin-O staining than the sham controls (Fig. 4A,B). Significant differences in gene expression were observed as well in the 1-month specimens. In all animals, BMP-2 could no longer be detected in the impact area, whereas cartilage from preserved areas outside the impact and from the sham limb was strongly positive (Fig. 4C). Also, the cells in the impact area no longer were positive for type II procollagen mRNA, indicating that these chondrocytes were not synthesizing new extracellular matrix. Chondrocytes within preserved areas and sham specimens were positive for type II procollagen mRNA (Fig. 4D). Many cells of the impact area (1-month post-impact) were positive for hematoxylin indicating the presence of nuclei (Fig. 4D). To determine if these intact cells retained the ability to synthesize protein, they were hybridized to a probe for 18S ribosomal RNA and were positive (data not shown). Positive 18S ribosomal RNA staining was a further indication that although these chondrocytes were not producing BMP-2 or type II procollagen mRNA, they were still viable.

At 6 months, specimens from each of the impact areas had evidence of cartilage degeneration. Abundant Safranin-O staining was present within preserved unimpacted areas, as well as the sham cartilage, but the impact areas were without Safranin-O staining (Fig. 5A,B). BMP-2 was detectable in the preserved areas, as well as the sham cartilage, but not within the area of impact (Fig. 5C). Unlike the 1-month specimens, there was no hematoxylin staining in the impacted area, and empty lacunae were detected (Fig. 5A). Neither the control specimens, nor the impacted specimens demonstrated synthesis of type II procollagen mRNA at 6 months, likely due to the ageing of the animals.
Figure 3. Photomicrograph of a Safranin-O–stained cartilage specimen from a High impact load animal at time 0 (A). BMP-2 staining of preserved, impacted, and sham cartilage (left to right; B), demonstrating little or no change in BMP-2 production. In situ hybridization of type II procollagen mRNA of preserved, impacted, and sham cartilage (left to right; C), demonstrating little or no change in in situ hybridization analysis of type II procollagen mRNA. Bar represents 100 μm.

Figure 4. Photomicrograph of a Safranin-O–stained cartilage specimen from a High impact load animal at 1-month postinjury (A). From left to right (B), higher power Safranin-O images of preserved, impacted, and sham cartilage. BMP-2 staining of preserved, impacted, and sham cartilage (left to right; C), showing considerable decrease in BMP-2 staining of the impacted specimen compared to the preserved and sham specimens. In situ hybridization of type II procollagen mRNA of preserved, impacted, and sham cartilage (left to right; D); note complete absence of type II procollagen mRNA in the impacted specimen as compared to the preserved and sham specimens. Bar represents 100 μm.
DISCUSSION

We have used our in vivo model of articular cartilage injury to investigate the effects of substantial impact loads on tissue morphology and chondrocyte metabolism in an attempt to determine whether a single high-energy impact load can stimulate the development of posttraumatic osteoarthritis. Using this model, we showed that cartilage damage does occur with impact loads of less than the fracture threshold for the condyle, and that these impact loads stimulate further deterioration of the tissue over time. Changes included a disruption of the cartilage matrix, loss of proteoglycan content, decreased cellular metabolism, and the eventual loss of chondrocytes, resulting in cartilage that has the appearance of posttraumatic osteoarthritis.

The molecular analyses reported here revealed two important characteristics of the animals used in this study. First, at the time of impact and at 1 month after impact, chondrocytes from these animals were very active, synthesizing abundant type II procollagen mRNA and BMP-2. However, at 6 months, the chondrocytes were no longer synthesizing type II procollagen mRNA. This result is likely due to the ageing of the animals during this 6 months following impact. At the time of impact, these young animals (3-months old) were actively growing and synthesizing abundant extracellular matrix. By the 6-month time point, the animals, now 9 months of age, were no longer actively making cartilage matrix. Secondly, by analysis of BMP-2 production as an indicator of chondrocyte metabolism, we determined that only the cells within the area of impact were responding to the injury; the chondrocytes in areas outside the impacted areas continued to demonstrate normal cellular activity.

In a previous study, we reported subtle changes in proteoglycan content in response to a relatively low impact load (45% of the fracture threshold). Others have also shown that the loss of matrix proteoglycan, similar to which occurs in early osteoarthritis, also has been shown to occur following impact load. It is unclear, however, whether these proteoglycans are lost into the synovial fluid due to mechanical disruption of the collagen or are lost as a result of enzymatic degradation without replenishment. In our previous impact study, we were unable to show enzymatic degradation of the proteoglycans.

BMP-2 has been shown to be an indicator for chondrocyte metabolic activity and was found to be up-regulated in human osteoarthritis. Additionally, the young rabbits studied in the current investigation also expressed BMP-2 production in their normal uninjured cartilage. However, with increased time from injury, BMP-2 production decreased only in the area of impact, and, at 6 months, was essentially absent within the area of impact. This implies that, as a result of subfracture threshold impact load, chondrocytes within the injured cartilage became considerably less metabolically active and theoretically less capable of repairing and maintaining ECM components and cartilage integrity. Our finding in young rabbits differs from the findings in human osteoarthritis at the time of joint replacement. Fukui et al. showed a dramatic up-regulation of BMP-2 production in human chondrocytes in intermediate and late stages of osteoarthritis, accomplished by increases in chondrocyte metabolism. This investigation also found that this up-regulation of BMP-2 could be induced by the proinflammatory cytokines, IL-1B and TNF-alpha.

Chondrocytes within the area of impact also showed a down-regulation of type II procollagen mRNA
production. This confirms the overall adverse effect of impact load on chondrocyte metabolism and further implies that cells in this region will be unable to repair and maintain the injured cartilage. Consequently, impact loading of growing active cartilage appears to inhibit normal cellular metabolism. It may be encouraging, however, to note that at least at 0- and 1-month time points, many cells retained intact nuclei and ribosomal RNA synthesis indicating that, for a time after injury, the cells retain some capacity for repair.

Generally, in the older human samples, anabolic activity within chondrocytes is very low; however, the process of primary OA involves the activation of chondrocyte metabolic activity. The young rabbits used in the present study are still metabolically active, as shown by the presence of BMP-2 and type II procollagen mRNA. In these young animals, the single high impact inhibited anabolic activity and this could provide a mechanism for further cartilage degeneration. Interestingly, in a previous study, we had shown no adverse effect of lower impacts (up to 45% fracture threshold) on cartilage. In preliminary studies designed to determine the differences in the cellular response between the lower impacts used previously and the higher impacts used in the current study, we found that, with lower impacts, BMP-2 levels were not reduced (Sandell, Franz, Borrelli, unpublished data) and thus the metabolic activity was essentially unaffected in rabbits of similar ages with less injury.

The limitations of utilizing techniques (histologic) to evaluate the effects of impact load on articular cartilage are apparent. These types of analyses allow only a description of the results and do not lean themselves to a quantitative analysis of the results. In order to minimize potential sources of experimental errors, meticulous attention was paid to using serial sections both from within, as well as outside, the area of impact and sham tissue. This allowed direct comparisons between different stained specimens. Additionally, sham specimens were analyzed from each of the animals, and adequate controls were utilized to confirm the accuracy of each of the staining processes.

In conclusion, it is apparent from this study that changes consistent with PTA can be initiated by the application of a single, subfracture, impact load. Changes were identified in each aspect of the cartilage including its morphology, ECM contents, cellular metabolism, and viability. These high impact loads, unlike the lower impact loads previously studied, caused inhibition of synthesis of major ECM molecules which would result in alterations in the cartilage’s mechanical properties, and these changes seem to be progressive. Initially, the cells lose the ability to synthesize matrix, as judged by collagen mRNA analysis, while retaining 18S ribosomal RNA and intact nuclei. These changes all advanced over time, culminating in cartilage degeneration at the impact site, indicating that a single high impact load can lead to cartilage degeneration.

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REFERENCES